SUMMARY AND CONCLUSIONS

1. The present studies have been undertaken to understand the manifestation of (a) the induced colony morphology variatiQn in the locally isolated strain of Rhodotorula glutinis CFR1 (b) to purify and characterise the asparaginase of Rhodosporidium toruloides CBS14 with the aim to understand its biological role and possible implication in morphogenesis. Related to this, the mode of asparaginase induction and physiological conditions for asparaginase biosynthesis in this yeast has been studied.

2. UV irradiation of Rh. glutinis CFR1 indicated typical dose related kill pattern with a small shoulder of resistance at the lower dose of UV. At the UV doses which do not apparently produce substantial kill (non-lethal dose) several colony morphology variants were observed. One of them, called Rg-Pink was subjected to detailed studies. The unirradiated parent culture produced colonies of uniform morphology and was termed as parental Smooth. It had a smooth surface and a perfectly circular border with a small protruberance in the centre. Among 70,000 colonies of the unirradiated culture screened, not a single colony morphology variant was detectable.

3. Rg-Pink originally observed as a slightly smaller colony than Rh. glutinis CFR1 with somewhat irregular border, on plating, gave rise to further colony morphology variants which were termed, Smooth-striated, Crater, Twisted and Beaded (Figs: 14, 15, 16 and 17). These were reasonably distinct and could be easily differentiated by visual observation.

4. In the experiment performed, to score the frequency of switch from one colony morphology variant to the other (as this kind of switch phenomenon has also been reported for Candida albicans (Ref. 102 of Review of Literature), the culture was repeatedly plated 18 times and 35,568 colonies were examined. No fixed frequency of switch of one form to the other similar to the reported pattern in C. albicans was seen. However,

In the overall assessment, the major proportion of the colonies were of the parental Smooth type (35%) followed by Beaded (28%), Crater (26%), Smooth-striated (6%) and Twisted (5%) types. The switch frequency variation in the overall plating was 0.87% to 86% for parental Smooth, 0 to 34.9% for Smooth-striated, 0.95% to 78.3% for Crater, 0 to 64% for Twisted and 2.1 % to 70% for the Beaded type.

5. The colony morphology variants were also plated to see the lineage of the colony types over seven platings. The parental Smooth was 78.8% in the third plating. It rose to 84.09% in the fourth plating and dropped to 35.42% in the fifth plating, when this colony morphology variant was plated repeatedly {Table 16). The Beaded type, similarly plated repeatedly, indicated 18.15% and 18.18% in the third and fourth plating, suddenly rose to 74.24% in the fifth plating, but dropped drastically to 4.03% in the sixth plating. The Crater type was 78.26% in the fourth plating, went down to 17.41 % in the fifth plating, rose to 63.02% in the sixth plating and again dropped to 18.88% in the seventh plating. This experiment involved the examination of 27,331 colonies. The conclusion was that no fixed lineage of the colony morphology type exists in this variant (Fig.18).

6. The manifestation of colony morphology variation in Rg-Pink at different growth phases during its growth in YEPD medium again did not indicate any distinct pattern or enrichment for a particular colony type at the end of the cultivation period reaching the late stationary phase of growth. In this experiment, a total number of 2,262 colonies were examined.

7. All the three experiments mentioned above indicated that there is no fixed frequency or distinct pattern of switch lineage and enrichment of the various colony types observed in the UV-induced variant, Rg-Pink.

8. When the Rg-Pink variant was compared with the parental CFR1 culture with respect to physiological and biochemical characteristics, no significant differences between the two cultures were detectable. Their growth kinetics were similar in different media under different pH conditions. Assimilation of various sugars, metabolic intermediates etc., were very much close. The only difference was found in the scanty growth of Rh. glutinis CFR1 and the luxuriant growth of Rg-Pink in the vitamin-free medium. This was eventually traced to a possible leaky phenotype for pantothenate requirement in the parental CFR-1 culture.

9. The cell morphology of Rg-Pink was examined microscopically in order to understand the manifestation of colony morphology variation observed in this culture. In contrast to the parental CFR1 culture which possess cells of uniform shape, and size (Fig.19I), the Rg-Pink was very much pleomorphic with respect to its cell morphology. On closer scrutiny, Rg-Pink was found to possess cells and structures resembling various stages in the well documented sexual cycle of Rhodosporidium yeast which is the perfect stage of Rhodotorula spp. (Fig.19A to H). Cells in conjugation, teliospores, dikaryotic mycelium etc., were distinctly discernible.

10. The major conclusions drawn from the study on colony morphology variation observed in Rh. glutinis CFR 1 by an apparent non-lethal dose of UV is that sexuality has been induced in the asexual imperfect Rhodotorula sp. and the only biochemical difference found in pantothenate requirement for the CFR1 culture and not in the variant Rg-Pink is attributable to either an independent mutational event or a transition from a leaky phenotype in CFR 1 to prototrophy in Rg-Pink by possible recombinational mechanisms to be expected during the sexual mode of reproduction in the latter.

11. Asparaginase is an enzyme implicated in the asexual sporulation process of the ascomycetous fungus Leptosphaeria michotii. An extensive study of the variation of asparaginase levels correlating with the circadian rhythm of

the sporulation process has been conducted (detailed in the Review of Literature). When Rhodotorula/Rhodosporidium cultures available in our laboratory (including Rh. glutinis CFR1) were examined for the presence of asparaginase, all of them were found to possess it. Since Rhodotorula/Rhodosporidium asparaginases have not been studied so far, it was intended to investigate the factors influencing the biosynthesis of this enzyme in a strain of Rhodosporidium toruloides CBS14 and also to purify and characterise the enzyme.

12. The enzyme was not detected in the culture fluid but was found only in washed cell suspensions which indicated that the enzyme was presumably located in the periplasmic space, which was later proven to be true by the following criteria.

    (a) Demonstration of fluorescence of intact cells with FITC-conjugate of antibody against anti-asparaginase antibody bound to cells suggested that the enzyme was cell bound.

    (b) Lack of agglutination by the anti-asparaginase antibody of whole cells indicated the absence of asparaginase bound externally to the cell wall.

    (c) The enzyme was freely accessible to the exogenous substrate and the non-requirement of detergents during the extraction process suggested that the enzyme was not membrane-bound, but possibly localised in the periplasmic space.

13. Rhodosp. toruloides CBS14 was capable of good growth in glucose, fructose and sucrose and somewhat less on xylose and mannitol with ammonium sulphate as the nitrogen source. Under these conditions no asparaginase was synthesised.

14. Asparaginase was produced by the organism only when asparagine was present in the medium. In the present case, asparagine could serve as sole source of carbon and nitrogen for the growth of the organism. Thus the asparaginase is an inducible enzyme.

15. Glucose at 110mM level caused carbon catabolite repression of asparaginase. At 10 fold lower levels of glucose (11mM), enzyme was induced by asparagine rather weakly in the beginning but strongly at a point, when an apparently complete utilisation of glucose had occurred.

16. Xylose which does not cause catabolite repression of the induction of asparaginase was utilised by the organism by a typical diauxic pattern, when both xylose and asparagine were supplied to the organism for growth. In this case asparagine was preferentially used first and xylose was used next with a distinct shift in the generation time.

17. The diauxic pattern of growth with xylose and asparagine is unique in the present case because both xylose and asparagine are utilised by inducible biochemical routes. The reason for the preferential utilisation of asparagine is understandably due to its capacity to serve as both a carbon and a nitrogen source. In a medium containing mannitol and asparagine also, an apparent diauxic pattern of growth was suggestive but not established.

18. Under all conditions of induced production of asparagmase, an accompanying glutaminase activity was found. Most strikingly, this was true in situations were glutamine was not at all supplied to the organism. In subsequent experiments, where several inducers were tested for asparaginase, asparagine, glutamine, aspartate and glutamate were all found to induce asparaginase activity. Although the levels of the enzymes based on specific activity) varied with different inducers, the ratio of asparaginase to glutaminase showed a similar trend with all the inducers tested.

19. Among the other inducers of asparaginase tested, beta-cyanoalanine and albizziin (both are aminoacids found in plants) were found to induce the asparaginase of Rhodosp toruloides CBS14. Enzyme action on these compounds was not confirmed. Both these compounds were found to be very poor supporters of growth of this organism.

20. Asparaginase II biosynthesis in Saccharomyces cerevisiae is known to be controlled by nitrogen catabolite repression. Rigorous experimentation failed to demonstrate this phenomenon in Rhodosp. toruloides CBS14. This is considered a distinguishing feature of the present enzyme.

21. Also unlike S. cerevisiae, Rhodosp. toruloides CBS14 possesses only a single asparaginase which is under multi-substrate induction as mentioned above.

22. By use of Ouchterlony double diffusion, the present asparaginase was found to be completely homologous with the asparaginases of Rh. glutinis CFR1 (which engendered Rg-Pink), Rh. glutinis NCYC59 and Rhodosp. toruloides ATCC10788. However, with Rh. rubra MTCC248 asparaginase, it was partially homologous (Fig.50A). The antibody of the present enzyme did not cross react with crude preparations of Serratia marcescens and Saccharomyces cerevisiae asparaginases.

23. Based on the induction pattern of asparaginase and its presence in the periplasmic space as a single enzyme, the present enzyme appeared to be unique.

24. Having determined that Rhodosp. toruloides CBS14 produces only one asparaginase localised in the periplasmic space (unlike more than one asparaginase reported in S. cerevisiae and E. coli) it was of interest to purify and characterise the enzyme.

25. Typically, for optimum production of enzyme, the yeast was cultured in liquid medium containing L-asparagine as the enzyme inducer (as well as source of nitrogen) and D-mannitol as an additional carbon source to the mid-exponential phase under wel1 aerated conditions at 30°C. Crude preparations of enzyme from the harvested cells subjected to acetone drying was obtained by extraction in phosphate buffer at pH 7 by grinding with glass beads.

26. In a typical experiment, starting from cel1s grown in 21 of culture medium, a crude enzyme preparation having 16,324 x 10^3 units of total asparaginase activity and 2,958 x 10^3 units of total glutaminase activity (with corresponding specific activities being 51.2 x 10^3 and 9.3 x 10^3 units/mg protein) in a total volume of 28ml was obtained. The purification steps, mostly conventional techniques, involved concentration by Sephadex G200, chromatofocusing, ammonium sulphate fractionation, gel filtration and preparative gel electrophoresis. This protocol led to a purified enzyme preparation with 19% recovery and 205 fold purification. with respect to asparaginase activity and with respect to glutaminase activity the recovery was 21.4% with a 232 fold purification factor. By SDS-PAGE and Ouchterlony double diffusion techniques, the final preparation seemed to contain a homogeneously pure protein.

27. The tendency of the enzyme to rapidly lose activity was considerably overcome by (i) including a protease inhibitor cocktail containing PMSF, benzamidine and EDT A in al1 the buffers and (ii) including 250 mM glycine and then adjusting the pH to 9. Some of the usual enzyme stabilisation techniques such as 20% glycerol and thiol group protecting agents were not helpful in the present case.

28. 28. The native molecular mass as determined by gel permeation in Sepharose-6B indicated it to be 180kDa. In the SDS-PAGE, it was found to be 87kDa suggesting that the enzyme was a homodimer. Enzyme obtained from cel1s in the stationary phase of growth, however, indicated the presence of an

additional 26.5kDa protein fragment detected by Western blotting using anti-asparaginase antibody. A cleavage product of this specific size was considered unique in this organism.

29. The enzyme was optimally active at pH 6.35 for both the L-asparaginase and L-glutaminase activities. The pH profiles for the two enzymes were however different, with the asparaginase activity showing a broader pH optima between 5 and 7. The glutaminase activity showed a sharp peak of activity at pH 6.35.

30. The temperature optima for both L-asparaginase and L-glutaminase activities was 37°C but the temperature related profiles of activity were different for the two activities.

31. The present enzyme's natural substrate, in all probability, is L-asparagine. Since it acts on L-glutamine and three other N-substituted asparagine analogs, the K_m values were obtained graphically. It was 1.43×10^{-3}M for L-asparagine, 6.45×10^{-3}M for L-glutamine, 10×10^{-3} for N-acetyl-L-asparagine, 8×10^{-3}M for N-carbamyl-L-asparagine and 2.4×10^{-3}M for N-glycyl-L-asparagine. It was evident that N-substitution of L-asparagine lowers the affinity of the enzyme for the substrate. L-glutamine which is apparently not the natural substrate also indicated a much lower affinity for the enzyme. The corresponding V_{max} values for the substrates mentioned in the same order as above were 133, 138, 364, 318 and 357 umoles/min./mg protein respectively.

32. Several metal ions and thiol group reagents and other functional group modifying reagents were not found to cause any significant change in catalysis, except the tryptophan modifiers, N-bromosuccinimide and 2-hydroxy-5-nitrobenzyl bromide which brought down the activity considerably suggesting the presence of tryptophan in the active site. This was further substantiated by substrate protection of the enzyme protein using asparagine or glutamine. Asparaginases having an essential

tryptophan residue in the active site have not been reported so far. Also, the asparagine analog, 5-diazo-4-oxo-L-norvaline (DONV) did not inhibit L-asparaginase activity.

33. By chromatofocusing, the pI of asparaginase was indicated as 8.6 by which it appeared similar to the bacterial asparaginases and very much different from the earlier reported yeast asparaginases.

34. By several criteria like the mode of induction, physico-chemical properties, substrate specificity etc., the present enzyme appears very unique. Its nutritional role is easily seen and the data suggested that there could be alternative routes in this organism for the utilisation of asparagine. The precise role of the present enzyme in morphogenesis could not be clearly pointed out with the available data. The asparaginase of Rhodosp. toruloides CBS14 showed complete homology with Rh. glutinis CFR1. The transition of the non-sexual to the sexual mode of reproduction correlated to molecular and biochemical events including the possible role of asparagine/asparaginase needs to be investigated further.